Lasers in Surgery and Medicine 21:255–261 (1997)

Pattern of Healing of Calvarial Bone in the Rat Following Application of the Erbium-YAG Laser

Mansour A. El Montaser, MSc,[1] Hugh Devlin, PhD,[1]* Philip Sloan, PhD,[2] and, Mark R. Dickinson, PhD[1]

[1]Department of Dental Medicine and Surgery, University Dental Hospital of Manchester
[2]Department of Physics, University of Manchester Manchester MI5 6 FH, England

Background and Objective: **The aim of this study was to examine the pattern of healing in rat calvarial defects prepared with the erbium-YAG laser, using the "guided tissue regeneration" technique [Dahlin et al., Scand J Plast Reconstr Hand Surg 1990;24: 13–19].**
Study Design/Materials and Methods: **PTFE membranes were placed over the lased skull defects and the skin wounds sutured. Rats were killed humanely at intervals after surgery and the skulls processed for paraffin wax histology. A further group of mature rats was killed humanely and the calvariae removed. Slots were prepared using the erbium-YAG laser and immediately examined under the environmental scanning electron microscope (ESEM) in hydrated conditions, which avoided drying artefact.**
Results: **An amorphous, mineral-rich carbon layer surrounds the lased bone defect, which in the *in vivo* experiments was seen as a basophilic zone that was resistant to resorption.**
Conclusion: **Bone infilling of the lased defect was retarded by delayed resorption of the amorphous, mineral-rich carbon layer. Lasers Surg. Med. 21:255–261, 1997.** **© 1997 Wiley-Liss, Inc.**

Key words: environmental scanning electron microscopy; guided tissue regeneration

INTRODUCTION

Previous studies have shown that laser ablation of bone is associated with secondary damage to the tissue surrounding the prepared defect [1]. The extent of secondary damage is variable and depends on laser type and energy. Erbium-YAG lasers tend to produce narrower zones of damage in bone than holmium-YAG lasers in vitro [2]. The bone composition and state of hydration also appear to be important variables [3]. Calvarial bone in vivo is a composite of mineralized and unmineralized matrix, cells, vessels and marrow, the latter often being rich in lipid-laden adipocytes. All of these elements may react differently to laser energy, and their degradation is likely to result in carbonization and exposure of bone mineral. The aim of the study was to examine the pattern of lased calvarial bone healing in a well-established rat model [4], using guided tissue regeneration to enable healing in critical size defects [5]. The pattern of long-term healing in erbium-YAG laser-ablated bone defects was examined. The study also aimed to investigate the nature of the zone of secondary damage in calvarial bone using scanning electron microscopy (SEM) methods that avoided fixation and drying artifacts (environmental SEM), together with conventional SEM. It was hypothesized that two of the important factors in determining the rate of bone healing around the defect would be the de-

*Correspondence to: Dr. Hugh Devlin, Dept. of Dental Medicine and Surgery, University Dental Hospital of Manchester, Higher Cambridge St., Manchester M15 6 FH, England.

Accepted 16 October 1996

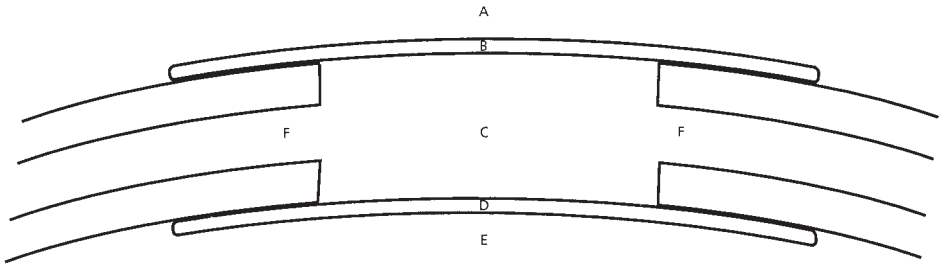

Fig. 1. Two layers of polytetrafluoroethylene membrane were used to exclude cells from the skin **(A)** and dural membranes **(E)** from gaining access to the lased defect **(C).** Membranes were applied to the outer and inner bone surfaces (**B** and **D**) so they overlapped the surrounding bone **(F)** by 3mm.

gree of carbonization and mineralization of the adjacent matrix.

MATERIALS AND METHODS

In Vivo Experiments

Seven mature Sprague-Dawley rats were anaesthetized with a gaseous halothane, nitrous oxide, and oxygen mixture. A 2 cm midline scalp incision was made and a periosteal flap was reflected. A circular 5 mm diameter full thickness disc of calvarial bone was outlined using two passes from a finely focused erbium-YAG laser beam in a handpiece. The disc of bone was gently mobilised and removed, carefully avoiding further trauma to the underlying tissue. The laser was used at 75 mJ/pulse with a lens of 50 mm focal length giving a fluence of 38J/cm^2. Ablated bone material and blood were removed by vacuum aspiration. Two layers of polytetrafluoroethylene Gore-Tex membrane (W.L. Gore and Associates Flagstaff, AZ) were used to cover the defect (Fig. 1)[6]. A membrane layer was gently placed on the inner aspect of the defect between the underlying dura and the inner table of bone. The defect was further covered on the ectocranial side, extending the membrane 3 mm beyond the defect onto the surface of the outer table. After haemostasis had been achieved, the outer membrane was covered by periosteum and temporalis muscle and sutured. Pairs of animals were killed humanely at 10, 20, and 30 days after surgery, and a further animal at 105 days after surgery. The calvariae were recovered, processed for routine wax histology, and stained with eosin and haematoxylin, and Lendrum's MSB.

In Vitro Experiments

Three mature Sprague-Dawley rats were killed and the calvariae removed. The erbium-YAG laser was used deliberately to apply a defocused beam to one bone surface (using 25 mJ/pulse or 13 J/cm^2). The beam was applied for a few seconds with the bone surface just beyond the focal plane of the lens. One slot was prepared in the outer surface of the other two calvariae using the erbium-YAG laser at either 50 mJ/pulse (25 J/cm^2) or 75 mJ/pulse (38 J/cm^2). The three calvariae were then examined immediately, without coating or drying in an electroscan E3 environmental scanning electron microscope (Wilmington, DE). X-ray analysis of the specimens was carried out to detect the presence of carbon in particular, using an ISIS system coupled to an atmosphere thin window detector (Oxford Instruments Microanalysis Group, High Wycombe, UK). Specimens were examined at 8°C and 6.4 torr, using an accelerating voltage of 20 kV.

A further six mature rats were killed and the calvariae removed. Three slots, a minimum of 3 mm apart, were made in each calvaria using two passes from the erbium-YAG laser at either 25, 50 or 75 mJ/pulse. The calvaria were freeze dried. For the scanning electron microscopy, the specimens were then coated with spectrographically pure carbon by evaporation in a Polaron E5000 12'' coating unit. Samples were examined in a Cambridge Instruments S360 scanning electron microscope (Leica Cambridge, Cambridge, UK) at an accelerating voltage of 25kV and a probe current of 500pA. The X-ray microanalysis was carried out using a Link Systems AN1000 multichannel analyser (Link Systems, High Wycombe, UK).

RESULTS

In Vivo Experiments

After 10 days, the calvarial defects had filled with granulation tissue, which included scattered chronic inflammatory cells. Collagen fibre

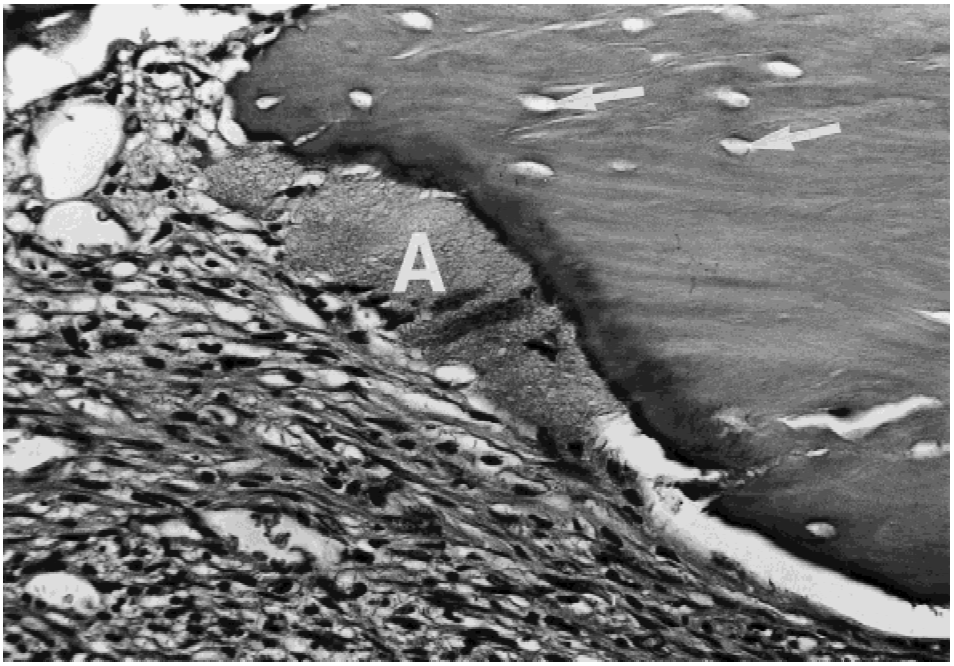

Fig. 2. Ten days after laser surgery showing amorphous material **(A)** covered by granulation tissue. Note empty osteocyte lacunae (arrows). (×100, haematoxylin and eosin).

bundles were abundant and small areas of woven bone were forming in the tissue. At the margin of the bone defect, the ablated surface was largely covered with a layer of amorphous haematoxyphilic material (Fig. 2). Osteocyte lacunae in the underlying bone appeared empty, suggesting loss of vitality. The granulation tissue attached to the amorphous material and in some places a gap was evident between the granulation tissue and bone due to either the amorphous material fragmenting or shrinkage artefact (Fig. 3). By 20 days, the calvarial defects were bridged by denser fibrous tissue. Woven bone had been deposited upon the margins of the ablated bone surface. The woven bone had formed over the amorphous layer and also on the endocranial surface. Sometimes a gap was evident between the newly formed woven bone and the ablated surface. A basophilic zone was apparent beneath the amorphous layer and osteocyte lacunae appeared empty.

At 30 days following laser ablation, the defect was bridged by mature fibrous tissue that contained discontinuous areas of remodelling woven bone. At the periphery of the defect, there was considerable deposition of woven bone (Fig. 3). The laser ablated bone had begun to sequestrate and was characterized by increased basophilia and empty lacunae. Undermining resorption had occurred and vital lamellar bone was forming on the outer surface (Fig. 3). The amorphous material still covered most of the ablated surface, where it appeared to prevent resorption and proper union of the woven repair bone. Occasionally there was continuity of the woven bone and ablated surface in places where the amorphous material was apparently absent.

In the specimen recovered after 105 days, the calvarial defect had not been bridged by repair bone. The ablated surface, largely covered by a fine amorphous layer, was still present (Fig. 4). A wedge-shape, rounded mass of new bone was attached to the periphery of the defect by ectocranial and endocranial apposition. There was no apparent union between the new bone and the ablated surface (Fig. 4). Most of the newly formed bone was lamellar, suggesting that the initially deposited woven bone had undergone remodelling. Nonvital bone was present beneath the repair bone.

In Vitro Experiments

Environmental SEM studies. In the specimen that had received the defocused erbium-YAG laser beam, there was extensive charring of bone around the defect. When this specimen was examined in the environmental SEM under hydrated conditions, there were raised plaques with smooth surfaces that appeared to have incorporated some microspheritic bone mineral (Fig. 5). Quantitative

Fig. 3. Thirty days after laser surgery. More abundant reactive woven bone is present **(A),** but remains separated from the underlying bone by amorphous material (arrows). Sequestration and remodelling of the necrotic bone is occurring **(R).** (×25, MSB).

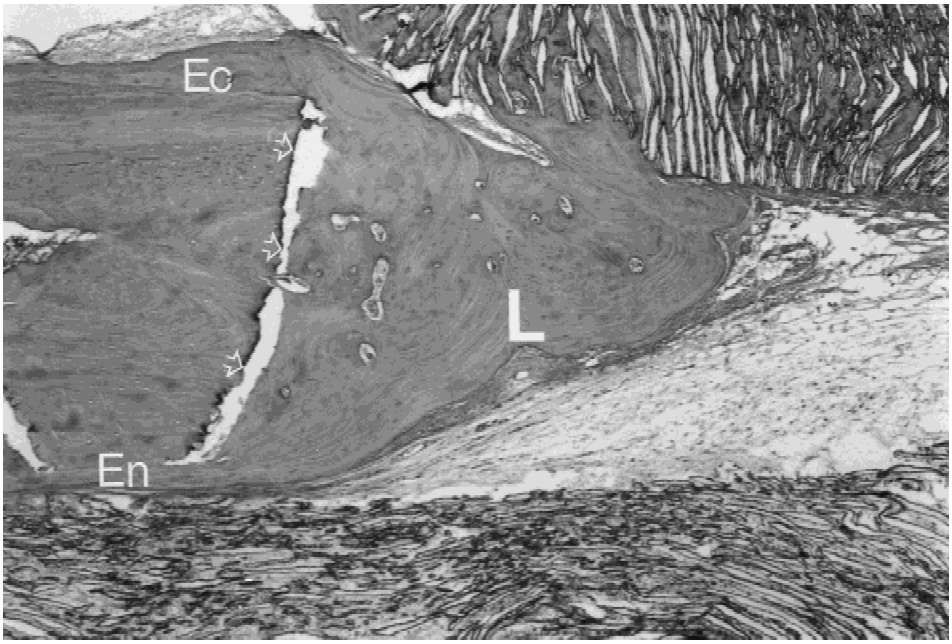

Fig. 4. 105 days after laser surgery. The woven bone has remodelled to form a wedge of lamellar bone **(L).** This is attached to the ectocranial **(Ec)** and endocranial **(En)** surfaces, but is separated from the margin of the lased defect (arrows). (×15.6, haematoxylin and eosin).

microanalysis of these areas indicated that they contained a high percentage of carbon. External to the ablated defect, pools of putative lipid material were apparent, intimately associated with the charred areas. The fluid material flowed under the environmental SEM conditions, but did not evaporate, indicating its nonhydrous nature. Beyond the lased area, microspheritic mineral particles were embedded in the bone surface.

Conventional SEM studies. In vitro ex-

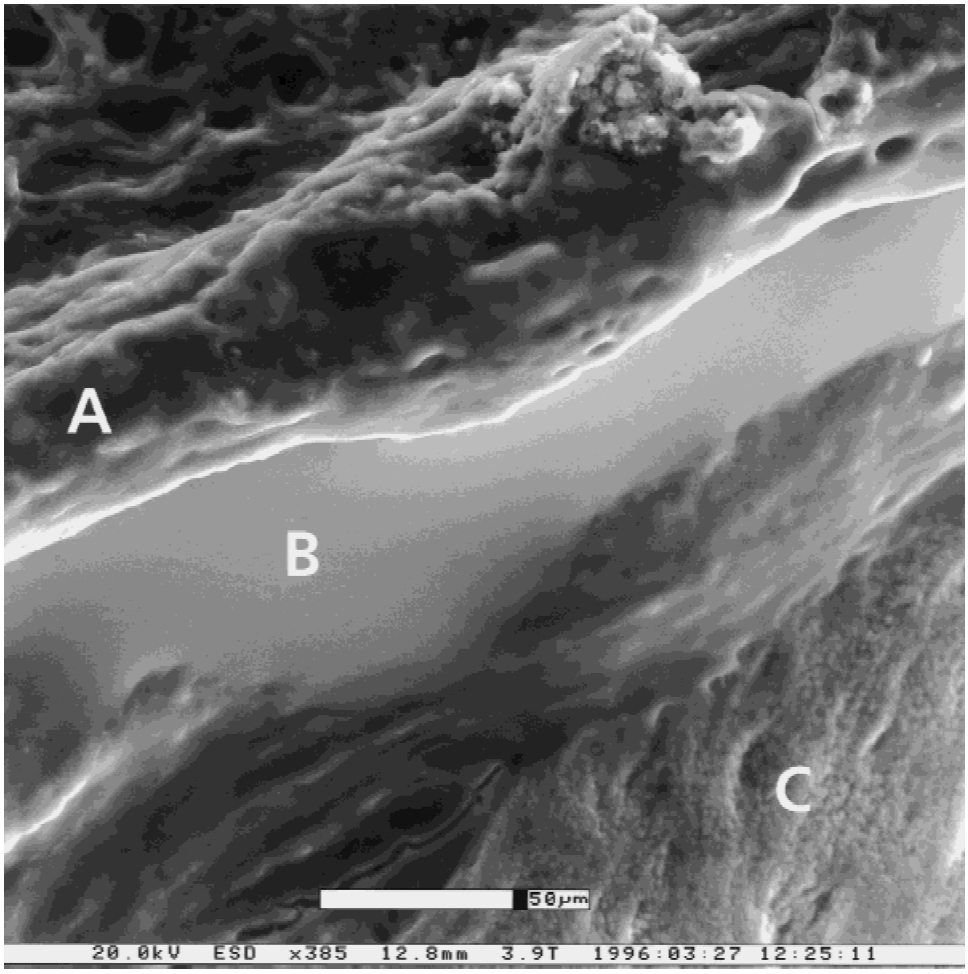

Fig. 5. Lased calvarial defect examined under hydrated conditions using the environmental scanning electron microscope. A smooth carbon layer, with encrusted mineral microspheres **(A)** is surrounded by a lipid fluid **(B).** The microspheritic pattern of the surrounding bone is also seen **(C).** ×385.

amination of the lased freeze-dried calvarial bone in the SEM showed that organic matrix was removed preferentially in a narrow zone around the laser prepared defect, exposing the mineralized matrix. In the back-scattered mode, a bright zone was present in the bone surrounding the lased defect, indicating a higher inorganic material content (Fig. 6A,B). The width of this zone varied with the energy per pulse, but was 80–110 μm wide in the slot prepared at 75mJ/pulse.

Quantitative X-ray microanalysis of the six calvariae at three representative sites on the edge of the 75 mJ/pulse lased defect indicated a mean % calcium of 27 (SD = 3.8) and % phosphate of 13.25 (SD = 2). However, at three other sites, 400 μm away from the slot, the % calcium and % phosphate content were lower at 20.63 (SD = 4.3) and 9.3 (SD = 2.7), respectively. The mean % calcium difference was significantly different at the two sites ($t = 5.23$, $P = 0.003$; CI = 3.24, 9.48), as was the mean % phosphate difference ($t = 6.59$, $P = 0.001$; CI = 2.4, 5.5). There was no significant difference in the calcium to phosphate ratio (Ca/P ratio) between the two sites ($t = 1.77$, $P = 0.137$; mean difference = 0.23, CI = −0.105, 0.57). Examination of the exposed surface of the lased defect showed that it consisted of a melted zone, with scattered microspheres of inorganic material.

DISCUSSION

In the in vivo experiments, the bone surrounding the ablated defect underwent gradual undermining resorption and creeping substitution (replacement of the necrotic bone by new vital bone) [7]. The amorphous material adjacent to the defect consisted of a highly mineralized carbon layer, which inhibited direct resorption of the defect wall and subsequent appositional bone re-

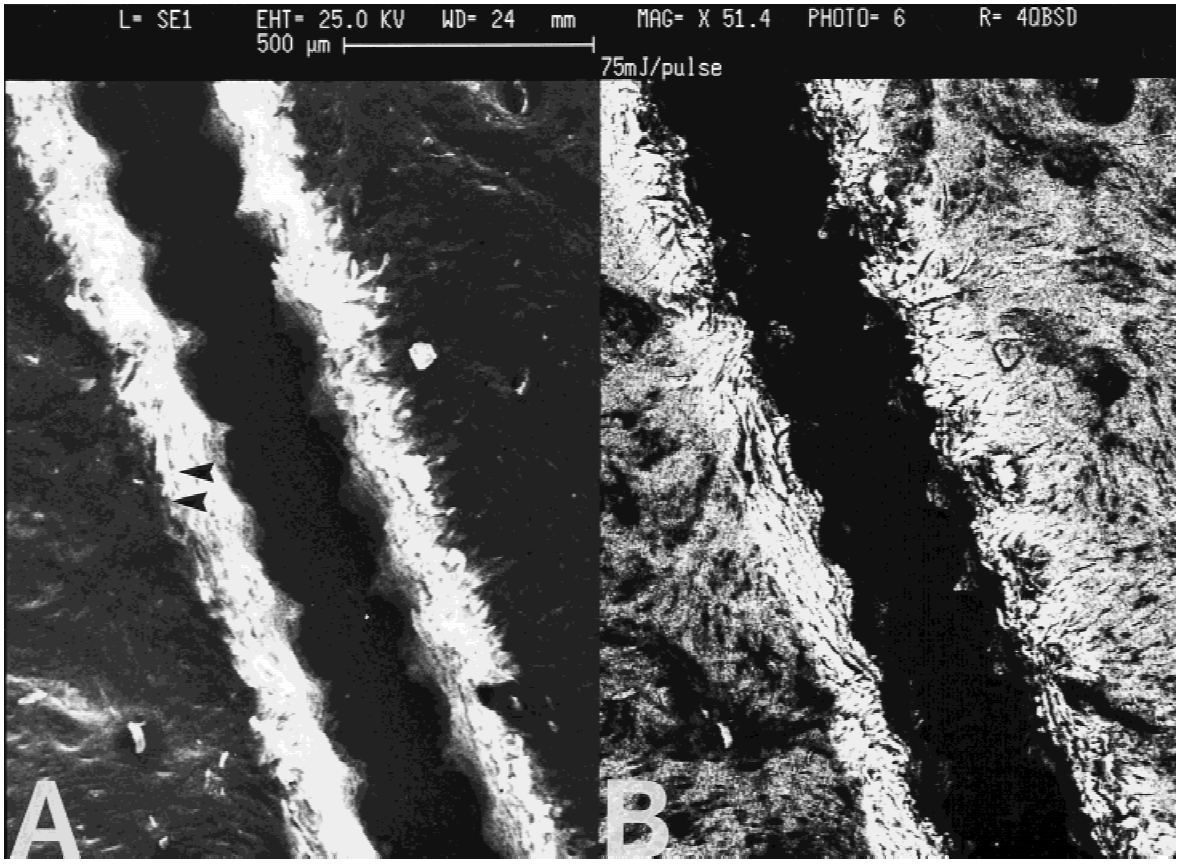

Fig. 6. **A.** The multilayered pattern of mineralized plates (arrowheads) in the lased slot (as observed in the conventional scanning electron microscope). ×51.4. **B.** In the back-scattered mode, a relatively highly mineralized layer was seen adjacent to the defect. The remainder of the calvarial surface was unevenly mineralized. ×51.4.

pair. Lasing of bone may cause carbonization either from heating of the organic bone matrix or disruption of the lipid-laden adipocyte layer in the marrow. The presence of the amorphous carbon layer may reduce osteoclast recruitment and inhibit direct resorptive activity.

Remodelling eventually resulted in incorporation of the carbonized mineral into the newly formed matrix. However, our studies indicated that this was a slow process as the amorphous layer persisted around the defect for 105 days, despite extensive remodelling of the surrounding necrotic bone. Previous studies have shown reduced bone formation following laser surgery, generally attributed to loss of bone forming cells [8]. Resorption of the necrotic bone is a necessary prerequisite for formation and attachment of the newly formed repair bone. Therefore, an inhibition of bone resorption may result in a decreased rate of bone formation following laser surgery.

Using guided tissue regeneration, mechanically prepared defects bridge completely with repair bone in 3–6 weeks in rat mandibles [9], 3–12 weeks in rat calvarial defects [6], and 6 weeks in rabbits [10]. The striking difference observed in the failure of the lased defects to bridge may be attributed to the failure of appositional repair. The sequence of repair events appeared similar, but healing stages were delayed in the lased defects. This delay may result in maturation of the fibrous bridging tissue to form a nonosseous scar. Such an end result would be less favourable in a clinical neurosurgical application.

Using an operating microscope and copious saline irrigation and suction, Lewandrowski et al.[11] found minimal thermal damage of bone using the erbium-YAG laser. They suggested that this laser could be used clinically to prepare precise holes in the thin bones of the maxillofacial region. In our study, the presence of extensive carbon deposits after application of the defocused laser beam emphasises the importance of controlling the fluence at the bone surface. With the lens used here, a deviation of only 2 mm away from the focal plane of the lens leads to a reduction in fluence of 50%. A reduction of this magnitude could easily take the interaction near to, or below, the threshold for photoablation, in which case the en-

ergy absorbed goes entirely into heating the tissue, and the formation of a carbon layer. Using the holmium-YAG laser, Wong et al.[3] found a selective removal of inorganic bone constituents, leaving collagen fibres at the base of the ablation crater. In our experiments, however, the erbium-YAG laser caused selective removal of organic material in a narrow zone adjacent to the laser defect, leaving the surface covered by single and clustered microspheres. These microspheres form a labyrinth of interconnected filaments. Pautard[12] described the calcium phosphate in bone matrix as being composed of such spherical particles (0.1–1 μm in diameter) and also more intricate, convoluted assemblies with dense cores joined by bridges. In the native state these calcified bridges surround collagen fibres [13]. These looped complexes were visible in the depth of the lased defect, suggesting that they were robust and relatively more resistant to ablation than the organic matrix.

The study has demonstrated that the formation of a carbon-mineral composite during erbium-YAG laser ablation may inhibit bone repair by guided tissue regeneration. Although exposure of bone mineral in vivo may stimulate bone repair [14], laser ablation results in loss of organic matrix associated with the mineral microspheres and consequent loss of biological activity. Our future studies will attempt to address both methods of reducing the extent of carbon-mineral composite formation and the possible introduction of matrix signals to stimulate repair.

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